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Characterization of Chicken Syndecan-3 as a Heparan Sulfate Proteoglycan and Its Expression during Embryogenesis

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Syndecan-3 is one of four identified members of a family of transmembrane proteoglycans (the syndecans) that possess highly similar cytoplasmic and transmembrane domains and may function as extracellular matrix receptors and/or low affinity receptors for signaling molecules such as FGF. We previously reported the cloning of a partial cDNA for chicken syndecan-3. Here we report the isolation of a syndecan-3 cDNA containing additional 5′ sequence which includes a potential methionine start codon and putative signal sequence. *In vitro* translation of syndecan-3 cDNA in the presence and absence of microsomes suggests that the putative signal sequence is functional, suggesting that the cDNA may encompass the full coding sequence. We also identify syndecan-3 as a heparan sulfate proteoglycan and report its expression pattern during chicken embryogenesis using polyclonal antibodies raised against a recombinant fusion protein. We detect abundant syndecan-3 expression in the developing brain and neural tube, including a striking expression in the floor plate of the neural tube. During limb development, syndecan-3 is expressed in the distal mesenchymal cells of the limb bud which are undergoing outgrowth in response to the apical ectodermal ridge. Syndecan-3 is also transiently expressed during the formation of the precartilage condensations of the skeletal elements of the limb and subsequently in association with the differentiating osteoblasts of the periosteum. Expression is also observed in several areas of tissue interactions including the developing lens, otic vesicle, genital ridge, sclerotome, and feather buds. © 1995 Academic Press, Inc.

INTRODUCTION

Embryogenesis proceeds through morphogenic movements which involve changes in cell shape, adhesion to extracellular matrices, and cell–cell interactions. During this process cells with different developmental histories become juxtaposed, allowing signals to be sent between two apposing tissues which act to further specify the fates of one or both tissues (Sanders, 1987; Wessells, 1977; Fleischmajer *et al.*, 1968). A number of recent studies have demonstrated the importance of heparan sulfate (HS) proteoglycans in cell-signaling events, including those mediated by members of the FGF family (Kan *et al.*, 1993; Olwin and Rapraeger, 1992; Salmivirta *et al.*, 1992; Rapraeger *et al.*, 1991; Yayon *et al.*, 1991). Syndecans are a family of four transmembrane proteoglycans which contain highly conserved transmembrane and cytoplasmic domains (Baciu *et al.*, 1994; David *et al.*, 1992; Pierce *et al.*, 1992; Kojima *et al.*, 1992; Carey *et al.*, 1992; Gould *et al.*, 1992; Marynen *et al.*, 1989; Saunders *et al.*, 1989) and share additional structural characteristics. Syndecan-1 (originally designated syndecan), the most extensively studied syndecan, may function as a receptor for a variety of extracellular matrix molecules and as a low-affinity receptor for growth factors such as FGF (Bernfield *et al.*, 1992). These functions are thought to occur through the interactions of the HS glycosaminoglycan (GAG) chains present on the core protein and are therefore not likely unique to syndecan-1 but may be shared by other syndecan family members as well as other HS proteoglycans. Thus, it has been suggested that syndecan-1, and other syndecan family members, may mediate the interactions of cells with extracellular components that control cell shape, adhesion, proliferation, and differentiation (David, 1993; Bernfield *et al.*, 1992). Syndecan-1 exhibits spatiotemporal and cell-type-specific patterns of expression during embryonic development (Bernfield *et al.*, 1992). During organogenesis, syndecan-1 is expressed in areas of epithelial–mesenchymal tissue interactions, and its expression has been shown to be regulated by these interactions (Vainio *et al.*, 1989a,b). In later phases of embryogenesis and into the adult, syndecan-1 expression becomes increasingly restricted to epithelial cells. Conversely, syndecan-2 (fibroglycan) has been recently shown to be expressed exclusively by mesenchymal tissues during embryogenesis, suggesting that these structurally related proteoglycans may exhibit distinct and/or complementary spatiotemporal expression domains (David *et al.*, 1993).

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Partial cDNAs for rat (*N*-syndecan, Carey *et al.*, 1992) and chicken syndecan-3 (Gould *et al.*, 1992) have been characterized. Syndecan-3, like other members of the syndecan family, contains highly conserved cytoplasmic and transmembrane domains and an extracellular domain which includes clustered potential GAG attachment sites and a protease-sensitive site near the transmembrane domain (Carey *et al.*, 1992; Gould *et al.*, 1992). Based on its sequence and overall structure, syndecan-3 is most closely related to syndecan-1 (Bernfield *et al.*, 1992; Gould *et al.*, 1992). However, syndecan-3, unlike other syndecans, contains a threonine-, serine-, and proline-rich (T–S–P) region in the extracellular domain of the molecule. This region, which resembles mucin proteins, has the potential to receive numerous oligosaccharide chains (Gould *et al.*, 1992), giving syndecan-3 an additional potential functional domain not found in other syndecans. Abundant transcripts for syndecan-3 have been detected in the developing rat central nervous system (Carey *et al.*, 1992) and in the chondrogenic central core of the chick limb (Gould *et al.*, 1992). Thus syndecan-3 has been suggested to be involved in the development of these tissues, functioning either as a cell–cell or cell–matrix receptor (Carey *et al.*, 1992; Gould *et al.*, 1992) or as part of a receptor complex for FGF-2 (Chernousov and Carey, 1993) or the heparan binding growth-associated molecule (HB-GAM) present in neonatal rat brain (Raulo *et al.*, 1994).

In the present work, we report the isolation of a chicken syndecan-3 cDNA containing additional 5′ sequence that encodes a potential methionine start codon and a putative signal sequence, identify chicken syndecan-3 as a heparan sulfate proteoglycan, and report the expression pattern of syndecan-3 in the developing chick embryo using polyclonal antibodies generated against a recombinant fusion protein. Syndecan-3 exhibits spatially discrete and developmentally regulated expression domains in the chick embryo which suggest it may play an important role in the morphogenesis and differentiation of several organ systems, particularly the developing central nervous system and limbs.

EXPERIMENTAL PROCEDURES

Isolation of Syndecan-3 cDNA and Genomic Clones

Overlapping cDNA clones pCon-1, pCon-2, and pCon-PCR and genomic clones were obtained as described (Gould *et al.*, 1992). pCon-3, a 726-bp clone, was isolated through screening a random-primed cDNA library in λ Bluemid using the 5′ end of pCon-PCR as a probe (Fig. 1; nucleotides 100–856 in Fig. 2). The library was prepared from embryonic Day 4–6 chick limb bud poly(A)$^+$ RNA denatured with mercuric hydroxide to increase the length of the cDNAs. Conventional screening, as described previously (Sandell *et al.*, 1983), was supplemented with the polymerase chain reaction (PCR) to identify plate lysates with clones containing sequences at the 5′ end of the previously reported sequence (primer pair 110–130; 444–428; Fig. 2). PCR was carried out essentially as previously described by Gould *et al.* (1992).

Northern Analysis

Northern blot analysis was performed using total RNA isolated by a modification (Nichols *et al.*, 1990) of the guanidine isothiocyanate/cesium chloride centrifugation procedure of Chirgwin *et al.* (1979). Northern blots were prepared as described by Sambrook *et al.* (1989) and then prehybridized, hybridized, and washed as previously described (Kosher *et al.*, 1986), except that hybridization and washing were performed at 50°C. Levels of actin mRNA were determined through subsequent hybridization of blots with a cross-hybridizing rat actin cDNA probe.

In Situ Hybridization

In situ hybridization was performed using a double-stranded cDNA probe (Fig. 1) essentially as described previously (Mallein-Gerin *et al.*, 1988).

In Vitro Transcription/Translation

An *Eco*RI/*Sma*I fragment of pCon-3 was cloned into *Eco*RI/*Sma*I-digested pCon-2, replacing the small *Eco*RI/*Sma*I fragment of pCon-2. The resulting clone, pCon-4, contains an open reading frame of 405 amino acids including a putative signal peptide at the N-terminus (amino acids 1–22). Sense RNA was transcribed from 4.5 μg of *Xba*I linearized pCon-4 plasmid using T7 polymerase according to the supplier's recommendations (Promega Corp., Madison, WI). *In vitro* translation reactions were performed in the presence of [^{35}S]-methionine using nuclease-treated rabbit reticulocyte lysate (Promega Corp.) according to the supplier's recommendations, with and without the addition of 5.0 μg (0.25 μg/μl) of sense pCon-4 RNA template as a substrate. Dog pancreatic microsomes (0.9 μl per 12.5-μl reaction) were added to some of the reactions in order to determine if the signal peptide predicted by the cDNA was able to be proteolytically cleaved. Trypsin, when added, was used at a final concentration of 0.1 mg/ml and incubated at 37°C for 45 min, with and without the inclusion of 0.1% Triton X-100 (Sigma Chemical Co., St. Louis, MO) to disrupt microsomes. Aliquots from reactions were loaded on a 10% SDS–PAGE gel essentially as described by Laemmli (1970). Fluorography was performed using Entensify (Dupont NEN Research Products, Boston, MA) as recommended by the manufacturer.

Protein Extraction and Fractionation

Tissue extracts were obtained by sonication on ice in phosphate lysis buffer containing 0.01 *M* sodium phosphate buffer, pH 7.4, 0.1 *M* NaCl, 1% Triton X-100, 0.1% SDS, 0.2% sodium deoxycholate, and 1 m*M* PMSF. An aliquot of tissue extract was precipitated with 4 vol of ice-cold methanol and resuspended in 8 *M* urea prior to protein determination via the Bradford method (Bradford, 1976).

Proteoglycans were extracted with 4 *M* guanidine-HCl, 50 m*M* sodium acetate, 10 m*M* EDTA, pH 6.0, with 2% Triton X-100, 0.1 *M* 6-amino-hexanoic acid, 10 m*M* *N*-ethylmaleimide, 5 m*M* benzamidine-HCl, and 1 m*M* PMSF essentially as described by Midura and Hascall (1989). Tissues were solubilized by either gentle end-over-end mixing at 4°C for 18 hr or by rapid homogenization in a Dounce homogenizer on ice. Samples were then clarified by centrifugation at 10,000*g* and exchanged to buffer A (8 *M* urea, 0.05 *M* sodium acetate, 0.15 *M* NaCl, pH 6.0, with 0.5% Triton X-100) over a Sephadex G-50 (Pharmacia LKB Biotechnology, Piscataway, NJ) column equilibrated with buffer A. Samples were then applied to a 2-ml Q-Sepharose ion-exchange column (Pharmacia LKB Biotechnology) and washed with 3 vol of buffer A. Moderately charged proteins and nonsulfated carbohydrates were washed from the column with 3 vol of buffer A containing 0.3 *M* NaCl, while proteoglycans and other highly anionic glycoproteins were then eluted, using 3 vol of buffer A containing 1.4 *M* NaCl (Yanagishita *et al.*, 1987). This proteoglycan-enriched fraction was then precipitated with 3 vol of ice-cold ethanol, dissolved in a small volume of 4 *M* guanidine-HCl, 50 m*M* sodium acetate, 10 m*M* EDTA, pH 6.0, and dialyzed to PBS, pH 7.4, or appropriate enzyme digestion buffer, and the protein content determined (Bradford, 1976).

Chemical and Enzymatic Deglycosylation

Heparan sulfate GAG chains were removed through enzymatic digestion with heparitinase (Seikagaku America Inc., Rockville, MD). Briefly, 50 μg of the 1.4 *M* NaCl Q-Sepharose fraction was digested in 200-μl reactions containing 2 mU of heparitinase in 50 m*M* Tris-HCl, pH 7.4, 10 m*M* sodium acetate, 5 m*M* EDTA, with 50 m*M* 6-aminohexanoic acid, 10 m*M* *N*-ethylmaleimide, 0.1 m*M* pepstatin A, 0.1 m*M* PMSF, 5 mg BSA, and 1 μg chondroitin sulfate A (Sigma Chemical Co.) for 3 hr at 37°C. Chondroitin sulfate GAG chains were removed through digestion with protease-free chondroitinase ABC (Seikagaku America, Inc.) in 50 m*M* Tris-HCl, pH 7.4, 10 m*M* sodium acetate, 5 m*M* EDTA, 10 m*M* *N*-ethylmaleimide, 0.1 m*M* PMSF for 3 hr at 37°C. Samples were also treated with trifluoromethansulfonic acid (TFMS) (Aldrich Chemical Co., Inc.) by a modification of the procedure described by El-Battari *et al.* (1987). Briefly, 50 μg of the 1.4-*M* NaCl Q-Sepharose fraction and 50 μg of ovalbumin were precipitated with 4 vol of methanol in glass vials with teflon-lined screw caps. To the dry pellets 60 μl of a 2:1 TFMS:Anisole (Aldrich Chemical Co., Inc.) mixture was added on ice under nitrogen and the vial sealed. Reactions were allowed to proceed for 2 hr on wet ice (0°C) with occasional mixing and were terminated by the slow, stepwise addition of 150 μl of 10 m*M* triethanolamine on a dry ice–acetone bath, followed by the addition of 30 μl of 1 *M* triethanolamine.

Polyclonal Antibody Production

Rabbit polyclonal antisera were generated through immunization with a recombinant syndecan-3 fusion protein. A region (696–1256) of pCon-2 coding for the C-terminal 186 amino acids of syndecan-3 was amplified by PCR using oligonucleotides containing *Bam*HI and *Eco*RI linkers. This fragment was cloned in-frame into the respective sites of the bacterial expression vector pGEX-2T (Pharmacia LKB Biotechnology). Bacterial cultures were grown in the presence of 1 *M* sorbitol and 5 m*M* betaine at 30°C in order to keep the fusion product in the soluble fraction of lysates (Blackwell and Horgan, 1991). The resultant fusion protein was partially purified using *S*-hexyl glutathione-agarose (Sigma Chemical Co.) with final purification over the hydrophobic interaction resin Octyl-Sepharose (Pharmacia LKB Biotechnology). Briefly, 1 liter of bacterial culture was pelleted and then lysed by sonication in 10 ml of PBS, pH 7.4, 10 m*M* EDTA, clarified by centrifugation, and brought to 1% Triton X-100. The sample was then incubated with 1 ml of *S*-hexyl glutathione-agarose (Sigma Chemical Co.) for 30 min at room temperature with gentle rocking, washed with PBS, pH 7.4, 10 m*M* EDTA, and eluted from the matrix by at least three separate 0.5 ml elutions with 50 m*M* Tris buffer, pH 7.5, 15 m*M* *S*-hexyl glutathione. Eluants were pooled and brought to 4 *M* guanidine-HCl, 0.05 *M* sodium acetate, pH 6.0 (buffer B), and incubated with 2 ml of Octyl-Sepharose (Pharmacia LKB Biotechnology) for 2 hr with gentle rocking. The resin was washed in buffer B and packed into a 2-ml column. Bound proteins were eluted with a 50-ml, 0 to 0.8%, linear gradient of Triton X-100 in buffer B. Fractions eluting at, or near, the inflection point of the column, as measured by absorbance at 260 nm (Yanagishita *et al.*, 1987), were pooled and precipitated with 4 vol of methanol. The pellet was solubilized in a small volume of buffer B and dialyzed to PBS, pH 7.4, prior to protein determination (Bradford, 1976) and assessment of purity by SDS-PAGE with visualization by Coomassie blue staining.

Preimmune sera were collected from two male White New Zealand rabbits (Millbrook Farms, Hadley, MA)

which were then immunized according to the following schedule: (a) initial immunization (Week 0) with 100 μg of intact fusion protein in complete Freund's adjuvant, administered intradermally; (b) subsequent booster injections at Weeks 2 and 4 with 100 μg of intact fusion protein in incomplete Freund's adjuvant, administered subcutaneously; (c) the initial round of bleeds at Weeks 6 and 8 were followed by repeating a cycle of Weeks 4 through 8. GST-adsorbed antisera were prepared by running immune serum over a GST affinity column containing the GST portion of the fusion product coupled to CNBr-activated Sepharose 4B according to the manufacturer's recommendations (Pharmacia LKB Biotechnology). IgG fractions were obtained from the GST-adsorbed antisera by selection on a protein A-Sepharose column (Pharmacia) as described by the manufacturer.

SDS-PAGE of Tissue Extracts and Proteoglycans

Polyacrylamide gradient gels (3.8–15%) were run essentially as described by Koda *et al.* (1985) except that gels were run on the Hoeffer Mighty Tall-Small single slab apparatus (Hoeffer Scientific Instruments, San Francisco, CA) at 60 V for 12 to 18 hr at 4°C.

Western Blot Analysis

Proteins were transferred to positively charged nylon membranes (MAGNAgraph Nylon, Integrated Separation Systems, Natick, MA), using the Bio-Rad Mini Trans-Blot Cell (Bio-Rad Laboratories) and the method of Towbin *et al.* (1979) at 70 V for 4 hr at 4°C. Membranes were equilibrated to TBS (0.02 *M* Tris-HCl, pH 7.5, 0.1 *M* NaCl) and then blocked in 3% gelatin in TBS for 3 hr at 37°C. Membranes were then processed for immunostaining as follows: (a) incubation in anti-syndecan-3 polyclonal antiserum (46–92 or 47–92) at 1:1000 in 1% gelatin TBS at room temperature (3 hr); (b) incubation in affinity-purified horse radish peroxidase (HRP) conjugated goat anti-rabbit IgG (Cappel/Organon Teknika Corp., Durham, NC) at 1:500 in 1% gelatin TBS at room temperature (30 min); (c) color development with 0.5 mg/ml diaminobenzidine (DAB) (Sigma Chemical Co.), 0.1% hydrogen peroxide (0.1% of a 30% stock) in TBS (5–20 min). Three 10-min washes in TBS-T (TBS–0.05% Tween 20) were performed following incubation in the primary and secondary antibodies. We employed the HRP-DAB detection method in order to maintain similar sensitivity conditions in our Western blotting and in our immunohistochemical analysis of syndecan-3 expression (see below).

Immunohistochemical Staining

The protocol used for these studies is a modification of that reported by Heine *et al.* (1987). Chick embryos or dissected tissues were prepared for immunohistochemical staining through fixation in Bouin's fluid except for tissues with excessive bone, in which case, Bouin's bone fixative was used. After fixation, tissues were dehydrated through a graded series of ethanols to 70% and stored at −20°C for variable periods. Prior to embedding in paraffin (Paraplast +, Oxford Labware, St. Louis, MO) tissues were completely dehydrated to absolute ethanol and the ethanol was replaced by xylene. Sections 5 μ*M* thick were deparaffinized and processed for immunohistochemical staining according to the following schedule: (a) blocking of endogenous peroxidase activity with 0.3% hydrogen peroxide in methanol (30 min); (b) treatment with 1 mg/ml hyaluronidase (Sigma Chemical Co., St. Louis, MO) in 0.1 *M* sodium acetate, 0.15 *M* NaCl, pH 5.5, at 37°C (30 min); (c) blocking of nonspecific protein binding with 10% normal goat serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in dilution buffer (0.02 *M* Tris buffer, pH 7.5, 0.1 *M* NaCl, 1% normal goat serum, and 0.1% BSA) (60 min); (d) incubation with anti-syndecan-3 fusion polyclonal rabbit antiserum (46–92 or 47–92) at 1:100 in dilution buffer at room temperature (60 min); (e) incubation in affinity-purified horse radish peroxidase conjugated goat anti-rabbit IgG (Cappel/Organon Teknika Corp.) at 1:40 in dilution buffer (60 min); (f) color development in 0.5 mg/ml diaminobenzidine (Sigma Chemical Co.), 0.015% hydrogen peroxide (0.015% of a 30% stock) in 0.02 *M* Tris-HCl, pH 7.5, 0.1 *M* NaCl (20–60 min); and (g) counterstain with hematoxylin. The buffer used for washes was 0.02 *M* Tris-HCl, pH 7.5, 0.1 *M* NaCl, 0.1% normal goat serum. Control sections were incubated with preimmune rabbit serum at 1:100 in dilution buffer and consistently showed no staining other than for patches of red blood cells whose endogenous peroxidase activity had not been quenched. GST-adsorbed IgG yielded essentially identical results as unpurified antisera and therefore unpurified antisera was used in the present study.

RESULTS

We have previously reported the cloning of a partial cDNA for chicken syndecan-3 (Gould *et al.*, 1992). In order to obtain additional cDNA sequence, we screened a random primed cDNA library prepared from Embryonic Day 4–6 chick limb poly(A)$^+$ RNA. A 726-bp clone (pCon-3, Fig. 1) that overlaps with previous clones and contains additional 5′ sequence was isolated. The clone contains a potential start methionine and a putative signal sequence (Heijne, 1986), suggesting that it may represent the N-terminus of syndecan-3. The predicted size of the syndecan-3 protein encoded by our composite cDNA is 405 amino acids with a molecular weight of 43,002 Da. The overall protein structure predicted by the composite cDNA includes a 22-amino-acid signal se-

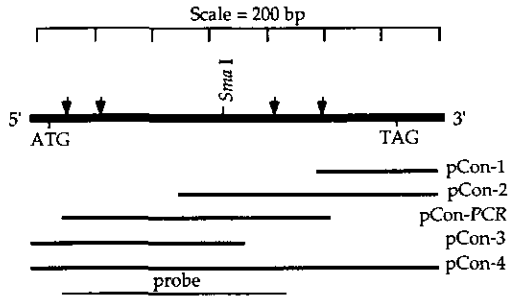

FIG. 1. Cloning map of syndecan-3. The composite cDNA is represented with arrows indicating exon boundaries. Clones pCon-1,2,3, and pCon-PCR have been described previously (Gould *et al.*, 1992). Start (ATG) and stop (TAG) codons are indicated. Probe indicates the region of pCon-PCR amplified by PCR (nucleotides 100–856 in Fig. 2) and used as a probe on Northern blots and for *in situ* hybridization.

quence followed by a 325-amino-acid extracellular domain, a 25-amino-acid transmembrane domain, and a 33-amino-acid cytoplasmic domain (Fig. 2). The extracellular domain contains a total of eight potential GAG attachment sites, divided into a N-terminal GAG cluster and a C-terminal GAG cluster, similar to the structure of syndecan-1 (Saunders *et al.*, 1989). Each of the potential GAG attachment sites consists of a SG dipeptide preceded and/or followed by acidic residues, resembling sites present within syndecan-1 (Saunders *et al.*, 1989). Comparison of the overall protein structure and amino acid sequence (Devereux *et al.*, 1984) with other syndecan family members suggests that syndecan-3 is most closely related to syndecan-1 (37% amino acid identity). Unlike syndecan-1, however, there are no potential sites for *N*-glycosylation present in the predicted amino acid sequence for chicken syndecan-3 (Saunders *et al.*, 1989). In addition, there are no cysteine or histidine residues. The region of syndecan-3 which most distinguishes this protein from syndecan-1 is the presence of a 182-amino-acid region which is 57% threonine, serine, or proline (T–S–P), located between the N-terminal and C-terminal GAG clusters (Fig. 2). Although not highly conserved at the amino acid level (42%), this region is also present in rat syndecan-3 (*N*-syndecan), consisting of a sequence rich in threonine and proline (Carey *et al.*, 1992).

In Vitro Translation

In order to determine if the potential translation start site and the putative signal peptide predicted by the composite syndecan-3 cDNA are functional, we expressed the protein *in vitro* using synthetic full-length syndecan-3 RNA as a template. Sense strand RNA transcripts from a composite cDNA (pCon-4, Fig. 1) were added to *in vitro* translation reactions containing rabbit reticulocyte lysate and [^{35}S]methionine. A syndecan-3

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1                                          GC CCC CGC GCG CTG CTG
18  AGC CGT CCT TGC GGC ACG AAG ATG CCC GCG GAG CTG CGG CGC
                                   Met Pro Ala Gln Leu Arg Gly  7

60  CTC GCG GTG CTG CTG CTG CTG CTC AGC GCC CGC GCA GCG CTG
    Leu Ala Val Leu Leu Leu Leu Leu Ser Ala Arg Ala Ala Leu  21

102 GCT CAG CGC TGG CGC AAT GAG AAC TAC GAG AGG CCG GTG GAC
    Ala Gln Arg Trp Arg Asn Glu Asn Tyr Glu Arg Pro Val Asp  35

144 CTG GAG GGC TCT GGG GAT GAT GAT CCC TTT GGG GAC GAT GAA
    Leu Glu Gly Ser Gly Asp Asp Asp Pro Phe Gly Asp Asp Glu  49

186 CTG GAT GAC ATC TAC TCG GGC TCC GGC TCA GGC TAT TTT GAG
    Leu Asp Asp Ile Tyr Ser Gly Ser Gly Ser Gly Tyr Phe Glu  63

228 CAG GAG TCA GGG TTG GAG ACA GCG GTC AGC CTC ACC ACG GAC
    Gln Glu Ser Gly Leu Glu Thr Ala Val Ser Leu Thr Thr Asp  77

270 ACG TCC GTC CCA CTG CCC ACC ACG GTG GCC GTG CTG CCT GTC
    Thr Ser Val Pro Leu Pro Thr Thr Val Ala Val Leu Pro Val  91

312 ACC TTG GTG CAG CCC ATG GCA ACA CCC TTT GAG CTG TTC CCC
    Thr Leu Val Gln Pro Met Ala Thr Pro Phe Glu Leu Phe Pro 105

354 ACA GAG GAC ACG TCC CCT GAG CAA ACA ACC AGC GTC TTG TAT
    Thr Glu Asp Thr Ser Pro Glu Gln Thr Thr Ser Val Leu Tyr 119

396 ATC CCC AAG ATA ACA GAA GCA CCA GTG ATC CCC AGC TGG AAA
    Ile Pro Lys Ile Thr Glu Ala Pro Val Ile Pro Ser Trp Lys 133

438 ACA ACC ACC GCC AGT ACC ACT GCC AGT GAC TCC CCC AGT ACC
    Thr Thr Thr Ala Ser Thr Thr Ala Ser Asp Ser Pro Ser Thr 147

480 ACC TCC ACC ACC ACC ACG GCT GCT ACC ACC ACC ACA ACC
    Thr Ser Thr Thr Thr Thr Thr Ala Ala Thr Thr Thr Thr Thr 161

522 ACC ACC ACC ATC AGC ACC ACT GTG GCC ACC TCC AAG CCC ACC
    Thr Thr Thr Ile Ser Thr Thr Val Ala Thr Ser Lys Pro Thr 175

564 ACT ACC CAG AGG TTC CTG CCC CCC TTT GTC ACC AAG GCA GCC
    Thr Thr Gln Arg Phe Leu Pro Pro Phe Val Thr Lys Ala Ala 189

606 ACC ACC CGG GCC ACC ACC CTG GAG ACG CCC ACC ACC TCC ATC
    Thr Thr Arg Ala Thr Thr Leu Glu Thr Pro Thr Thr Ser Ile 203

648 CCT GAA ACC AGT GTC CTG ACA GAG GTG ACC ACA TCA CGG CTT
    Pro Glu Thr Ser Val Leu Thr Glu Val Thr Thr Ser Arg Leu 217

690 GTC CCC TCC AGC ACA GCC AAG CCG AGG TCC CTG CCA AAA CCA
    Val Pro Ser Ser Thr Ala Lys Pro Arg Ser Leu Pro Lys Pro 231

732 AGC ACT TCC AGG ACT GCA GAA CCC ACG GAA AAA AGC ACT GCC
    Ser Thr Ser Arg Thr Ala Glu Pro Thr Glu Lys Ser Thr Ala 245

774 TTG CCT TCC AGC CCC ACC ACG CTG CCA CCC ACA GAA GCC CCC
    Leu Pro Ser Ser Pro Thr Thr Leu Pro Pro Thr Glu Ala Pro 259

816 CAG GTG GAG CCA GGG GAG TTG ACG ACA GTC CTC GAC AGT GAC
    Gln Val Glu Pro Gly Glu Leu Thr Thr Val Leu Asp Ser Asp 273

858 CTG GAA GTC CCA ACC AGT AGT GGC CCC AGC GGG GAC TTC GAG
    Leu Glu Val Pro Thr Ser Ser Gly Pro Ser Gly Asp Phe Glu 287

900 ATC CAG GAG GAG GAG GAG ACA ACT CGT CCT GAG CTG GGC AAT
    Ile Gln Glu Glu Glu Glu Thr Thr Arg Pro Glu Leu Gly Asn 301

942 GAG GTG GTG GCA GTG GTG ACA CCA CCA GCA GCA CCG GGG CTG
    Glu Val Val Ala Val Val Thr Pro Pro Ala Ala Pro Gly Leu 315

984 GGC AAG AAT GCA GAG CCG GGG CTC ATC GAC AAC ACA ATA GAG
    Gly Lys Asn Ala Glu Pro Gly Leu Ile Asp Asn Thr Ile Glu 329

1026 TCG GGC AGC TCG GCT GCT CAG CTC CCC CAG AAA AAC ATC CTG
     Ser Gly Ser Ser Ala Ala Gln Leu Pro Gln Lys Asn Ile Leu 343

1068 GAG AGG AAG GAA GTG TTG ATA GCT GTG ATT GTC GGC GGT GTG
     Glu Arg Lys Glu Val Leu Ile Ala Val Ile Val Gly Gly Val 357

1110 GTG GGA GCC CTC TTT GCT GCC TTC CTT GTC ATG CTG CTC ATC
     Val Gly Ala Leu Phe Ala Ala Phe Leu Val Met Leu Leu Ile 370

1152 TAC CGG ATG AAG AAG AAG GAC GAG GGC AGC TAT ACA TTG GAG
     Tyr Arg Met Lys Lys Lys Asp Glu Gly Ser Tyr Thr Leu Glu 385

1194 GAA CCC AAA CAA GCC AAC GTG ACC TAC CAG AAG CCG GAC AAG
     Glu Pro Lys Gln Ala Asn Val Thr Tyr Gln Lys Pro Asp Lys 399

1236 CAG GAG GAG TTC TAT GCG TAG GAC GAG AGC CCG GCA TGC CTC
     Gln Glu Glu Phe Tyr Ala *                              405

1278 ATG CTC CCT GCT CCT GCT CCA AAC TCT CCC TCC TCT CCT TCC
1320 ATC CCG TCT CTT TAT CCC TCT TCC CTT TTG GAT CAG ACT GTG
1362 AAT TTA AAA AGC
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FIG. 2. Nucleotide and deduced amino acid sequence of syndecan-3. The transmembrane region is indicated by underlining, while the threonine-serine-, and proline (T–S–P)-rich region is enclosed within brackets. Exon boundaries identified in the previously described genomic clones are indicated by closed triangles and the dibasic protease site by arrows. The open triangle represents the predicted site for signal peptide cleavage, while the open circles indicate serine residues which are potential sites for GAG attachment.

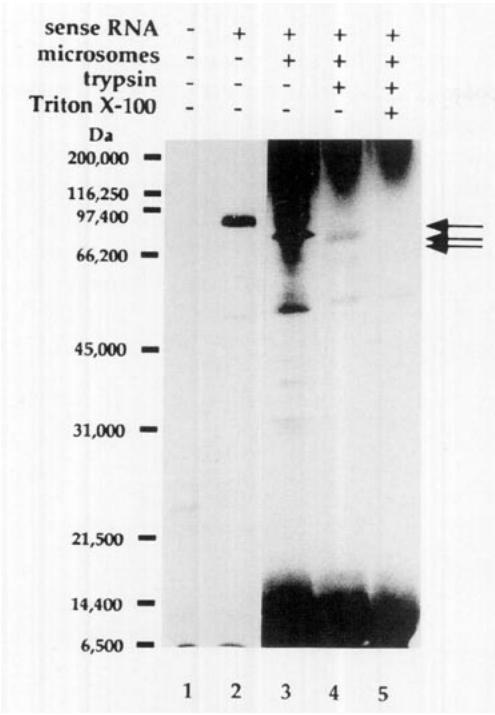


FIG. 3. *In vitro* translation of synthetic syndecan-3 RNA. A fluorograph of reaction mixtures incubated in the presence of [^{35}S]-methionine is shown. Lane 1, reaction with no RNA added. Lane 2, reaction containing sense syndecan-3 transcripts and no microsomes. Reactions in lanes 3-5 were performed in the presence of microsomes. Lanes 4 and 5 contain aliquots from the reaction loaded in lane 3 which were subsequently incubated with trypsin in the absence (lane 4) and presence (lane 5) of Triton X-100 in order to disrupt microsomes.

RNA template-dependent polypeptide was identified by SDS-PAGE and fluorography with an apparent electrophoretic mobility of 88 kDa (Fig. 3). Addition of microsomal membranes to the translation reactions caused a shift in the mobility of the polypeptide to approximately 77 kDa (Fig. 3), suggesting that proteolytic processing of the signal peptide by microsomal-associated proteases may have occurred (Walter and Blobel, 1983). It should be noted, however, that if only the putative signal sequence was being cleaved in the presence of microsomes, one might expect a product with a size of about 85 kDa, not 77 kDa as we observed. The reason for this discrepancy is not clear but may reflect the anomalous slow migration of membrane-spanning polypeptides observed on SDS-PAGE (Segrest and Jackson, 1972). At least some of the product is resistant to trypsin treatment, suggesting that it has indeed been translocated into microsomes (Fig. 3, lane 4). Consistent with this possibility, the product is degraded by trypsin in the presence of Triton X-100, which should disrupt the integrity of microsomes (Fig. 3, lane 5). The slight decrease in the size of the 77-kDa product in the presence of trypsin is consistent with the presence of a short cytoplasmic tail in the syndecan-3 protein, which would be predicted to be sensitive to trypsin treatment. It should also be noted that a band of about 50 kDa is also detectable in reaction mixtures in the presence and absence of microsomes. Although it is conceivable that this band may result from the presence of proteolytic activity in the preparations, it may well represent an artifactual band that is often seen in *in vitro* translation reactions done in the presence of [^{35}S]methionine (Jackson and Hunt, 1983; Sambrook *et al.*, 1989).

The predicted molecular weight of the syndecan-3 protein based upon the cDNA sequence is 43,002 Da prior to signal peptide cleavage and 40,759 Da after processing to the mature form. This differs from the apparent molecular weights of syndecan-3 produced *in vitro*, 88 and 77 kDa for unprocessed and mature syndecan-3, respectively. A similar discrepancy between predicted and observed molecular weights has been observed for all syndecans which have been described and has been attributed to the high alanine and proline content of these proteins which may result in regions of extended conformation leading to an overestimation of molecular weights by SDS-PAGE (Bernfield *et al.*, 1992, and references within).

Production of Polyclonal Antisera and Western Analysis

We have generated two polyclonal rabbit antisera (46-92 and 47-92) against a bacterial fusion protein. The fusion protein, produced using the pGEX-2T vector, contains the C-terminal 186 amino acids of the syndecan-3 protein fused to the N-terminal 223 amino acids of glutathione *S*-transferase (GST). Soluble fusion protein was purified through successive chromatography over an *S*-hexyl glutathione affinity column, selecting for molecules which contain glutathione *S*-transferase, and a hydrophobic interaction resin, selecting for molecules with extended hydrophobic regions, such as the transmembrane region of the syndecan-3. The apparent electrophoretic mobility of the fusion product, 64 kDa, was larger than the predicted 49 kDa based upon the amino acid sequence of the fusion protein (data not shown). The size discrepancy is consistent with the anomalous migration of the *in vitro* translated products on SDS-PAGE (see above). The fusion protein is recognized by both polyclonal antibodies on Western blots (data not shown).

As shown in Fig. 4, polyclonal antiserum 46-92 recognizes a broad band centered at 170 kDa present in homogenates of Embryonic Day 20 chick brain. An identical broad band is detectable with polyclonal antiserum 47-92 and with purified 46-92 IgG which has been passed over a GST affinity column to remove immunoglobins which recognize the GST portion of the fusion protein, indicating that the immunoreactive material is recognized by immunoglobins generated against the synde-

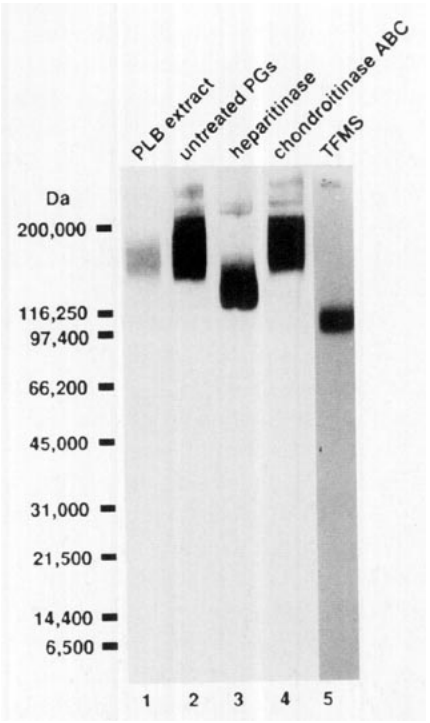


FIG. 4. Western blot analysis of Embryonic Day 19 brain extract, lane 1, or partially purified proteoglycans (under Experimental Procedures) from Day 20 brain, lanes 2–5. Lane 1 contains 250 μg; lanes 2–4, 25 μg; and lane 5 contains 10 μg as determined by the Bradford assay (Bradford 1976). Materials run in lanes 1 and 2 were untreated. Samples in lanes 3 and 4 were enzymatically digested with heparitinase or protease-free chondroitinase ABC, respectively. Material run in lane 5 was treated with trifluoromethansulfonic acid (TFMS). Incubation in primary antisera 46-92 at 1:1000 was performed for 3 hr at room temperature. Antisera 47-92 yields essentially identical results (data not shown).

can-3 portion of the fusion protein (data not shown). Transcripts for syndecan-3 are abundant in similar stage chick brain confirming that syndecan-3 is expressed in this tissue (Fig. 5). No bands are detectable with either antibody (46-92, 47-92) in extracts of embryonic liver which does not express detectable syndecan-3 mRNA (Gould *et al.*, 1992) nor are any bands detected in any tissue tested with the preimmune sera from the two rabbits in which the polyclonal antibodies were generated (data not shown). Treatment of partially purified proteoglycans (see Experimental Procedures) from Day 19 brain with heparitinase results in a broad band at 120 kDa, indicating that the immunoreactive material contains heparan sulfate GAG chains (Fig. 4, lane 3). Nitrous acid digestion yields essentially identical results (data not shown). Digestion with chondroitinase ABC did not alter the mobility of the immunoreactive material, indicating that syndecan-3 in Day 19 brain does not contain detectable amounts of chondroitin sulfate (CS) (Fig. 4, lane 4). Treatment with TFMS (El-Battari *et al.*, 1987) results in an increase in the mobility of the immunoreactive material to a broad band of approximately 110 kDa, suggesting the presence of carbohydrate not susceptible to heparitinase digestion (Fig. 4). A broad band of approximately 110 kDa is also detectable following TFMS treatment of partially purified proteoglycans extracted from Day 5 embryonic chick heart and limb buds, which are also sites of syndecan-3 transcript expression detectable by Northern blotting (Gould *et al.*, 1992; Fig. 5A). The broadness of the bands detectable after TFMS treatment suggests incomplete removal of carbohydrate, indicating that 110 kDa is an overestimate of the size of the core protein.

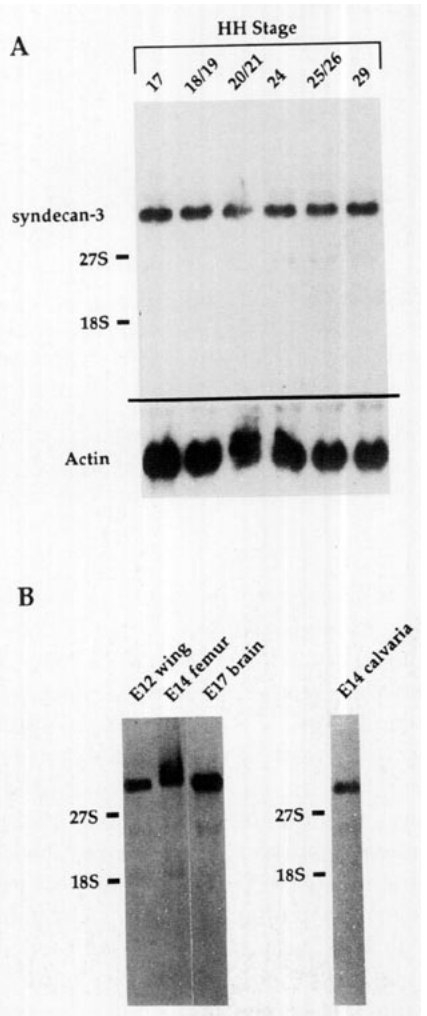


FIG. 5. Northern blot analysis of syndecan-3 mRNA expression. (A) Total RNA isolated from wing buds of various stage embryos showing approximately equivalent amounts of syndecan-3 transcripts during these early stages. A major specific 7-kb band is observed as well as a faint band below the 27S ribosomal band which likely represents an artifact frequently observed when loading total RNA. Hybridization with a cytoskeletal actin probe serves as a control for the amount of RNA loaded in each lane (10 μg). (B) Total RNA from Day 12 wing, the latest stage examined by immunohistochemistry, and osteogenic tissues demonstrating an association between syndecan-3 expression and osteogenesis. In addition there is abundant hybridization to RNA isolated from Day 17 brain. 10 μg of total RNA is loaded in each lane.

Expression of Syndecan-3 during Embryogenesis

We have surveyed the distribution of syndecan-3 in the chick embryo at selected stages and tissues between Days 1.5 and 20 of embryonic development by immunohistochemistry. At stage 12 (Hamburger and Hamilton, 1951) (Embryonic Day 1.5), the earliest stage examined, syndecan-3 immunoreactivity is most prominent in the head mesenchyme surrounding the optic vesicles (Fig. 6A). Slight staining is evident in the neuroepithelium of the mesencephalon with little or no staining associated with the prosencephalon (Fig. 6A). By stage 15 (Day 2), syndecan-3 immunoreactivity has become more intense especially in the head mesenchyme surrounding the optic cups and in the mesenchyme of the branchial arches (Fig. 6B). The placodal epithelia of the lens primordia and otic vesicles are also immunoreactive for syndecan-3 at this stage (Figs. 6C and 6D). In contrast, syndecan-3 is not detectable in the preplacodal ectoderm which gives rise to the otic and lens vesicles (data not shown). In the trunk of the embryo at stage 15, syndecan-3 is expressed in the sclerotome and in lateral plate mesoderm (Fig. 6E). The developing lens continues to express syndecan-3 at stage 25 (Day 5) with immunoreactivity localized to the developing lens fibers with more abundant expression posteriorly (Fig. 6F). At stage 31 (Day 7), we detect intense syndecan-3 expression in both the mesoderm and ectoderm of the genital ridge (Figs. 6G and 6H).

Expression of syndecan-3 in the neuroepithelium can be seen throughout the stages examined, becoming most prominent upon the formation of morphologically distinguishable axons. At stage 25 (Day 5), the neural tube consists of several distinct cell layers, and syndecan-3 expression predominates in the medial and marginal zones, both regions rich in axonal processes, with less staining in the ventricular layer (Fig. 7A). Syndecan-3 immunoreactivity is more abundant in the ventral half of the neural tube, including a striking expression in the floor plate (Fig. 7A). Considerably later in development (Day 20), syndecan-3 immunoreactivity is widespread in the cortex and cerebellum of the brain (Figs. 7C and 7E). Expression appears uniform throughout these areas except for being absent in the ectodermal coverings and particularly strong in the basal ganglia (Fig. 7C). Syndecan-3 protein detectable by Western blotting and mRNA detectable by Northern blotting are abundant in the brain at similar stages of development (Figs. 4 and 5). Sections incubated with preimmune antisera reveal no immunoreactivity (Figs. 7A and 7D).

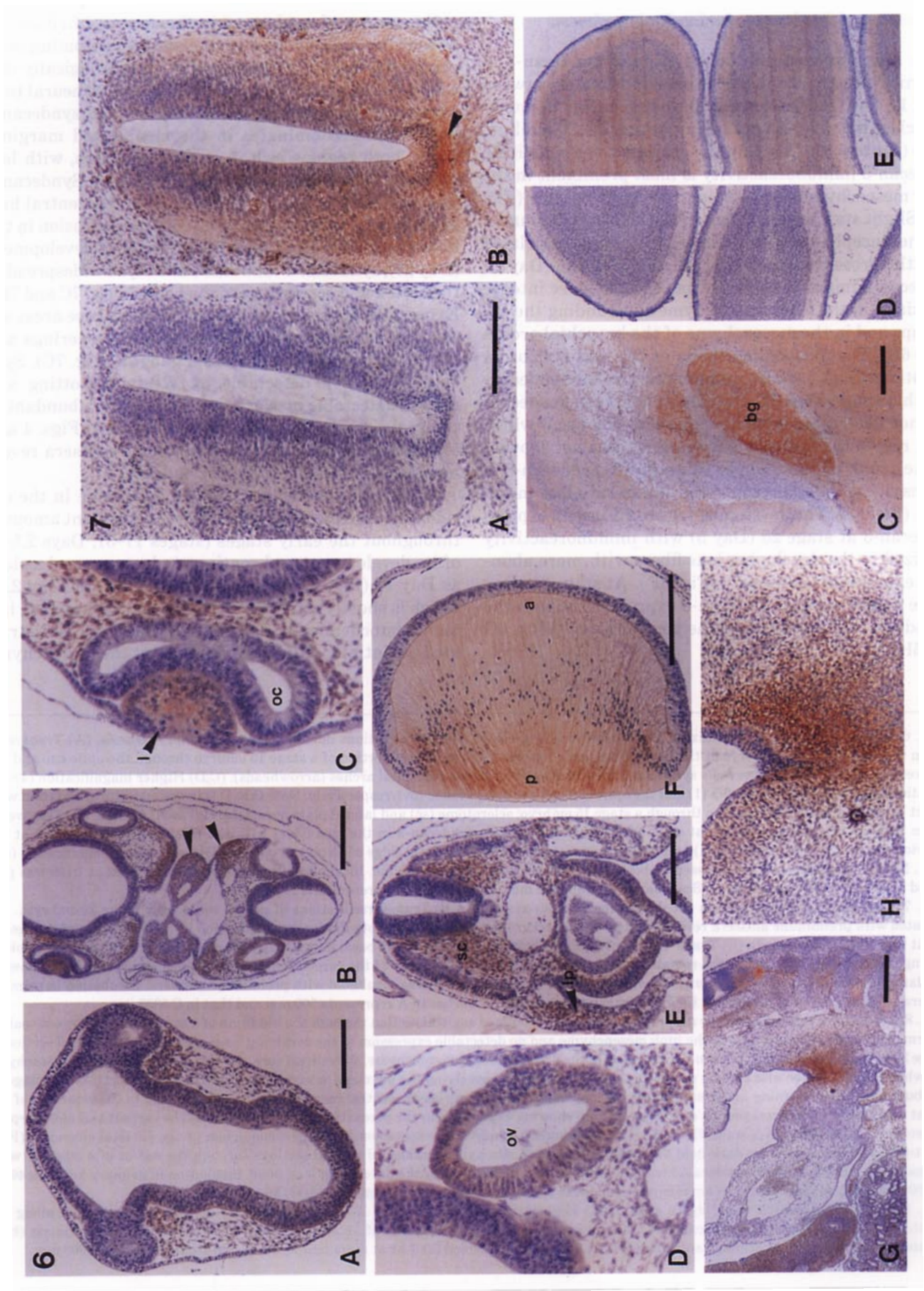
Transcripts for syndecan-3 are detectable in the developing limb bud at approximately equivalent amounts throughout the early stages (stages 17–31, Days 2.5–6) of its development and continue to be expressed as late as Day 14 (Figs. 5A and 5B). At stage 17/18 (Day 2.5), which is shortly after the formation of the limb bud, immunohistochemically detectable syndecan-3 is distributed essentially uniformly throughout the mesenchyme

FIG. 6. Immunohistochemical localization of syndecan-3 in areas of tissue interactions during early chick embryogenesis. (A) Transverse section through a stage 12 embryo at the level of the optic vesicles. (B) Transverse section of a stage 15 embryo through the optic cup and the optic vesicle. This section also reveals syndecan-3 immunoreactivity in the branchial arches (arrowheads). (C,D) Higher magnification (4×) of the optic cup (oc) and lens primordia (l) and the optic cup and the optic vesicle (ov), respectively. Note that D is in the opposite orientation with respect to B. (E) A transverse section through a stage 15 embryo; sclerotome (sc) and lateral plate mesoderm (lp) are indicated. (F) Transverse section through the developing lens at stage 25 indicating abundant syndecan-3 in the lens fibers with the most intense expression at the posterior margin (p; a, anterior). (G,H) Longitudinal sections through the genital ridge of a stage 31 embryo; (H) is a higher magnification (4×) of (G). Note that syndecan-3 is expressed by both the ectoderm and the mesenchyme. Incubation in primary antisera 46–92 at 1:100 was performed for 1 hr at room temperature. Scale bars represent 500 mm, except in G which represents 1000 mm.

FIG. 7. Expression of syndecan-3 in the developing nervous system. (A,B) Transverse sections of neural tubes from stage 25 embryos. (A) Incubated with preimmune antisera revealing little or no background staining. (B) Note the intense expression in the floor plate of the neural tube at this stage (arrowhead). (C) Transverse section of a Day 20 brain showing the basal ganglia (bg) heavily stained with elevated levels of staining uniformly distributed in the remaining tissue. (D,E) Transverse sections of the cerebellum from the same embryo as in C, showing abundant but essentially uniform expression of syndecan-3. The section in D was incubated with preimmune antisera. Incubation in primary antisera 47–92 at 1:100 was performed for 1 hr at room temperature. Scale bar in A represents 500 mm and that in C 1000 mm.

FIG. 8. Syndecan-3 expression in the developing limb. (A) An oblique sagittal section through the hindlimb of a stage 17/18 embryo revealing uniform expression of syndecan-3 in the limb mesenchyme and no detectable expression in the overlying limb ectoderm. (B) Sagittal section of a stage 22 wing bud revealing syndecan-3 expression in the distal subridge mesenchyme, the central core, and the proximal ventral mesenchyme (arrowhead). This section also reveals ectodermal immunoreactivity overlying the proximal ventral ectoderm. (C) Frontal section of a stage 25 wing bud illustrating staining in the subridge mesenchyme and the chondrogenic central core. No staining is observed in the ectoderm of the limb at this stage. (D) Frontal section of a stage 28 limb showing expression in the precartilage condensations of the carpals and metacarpals. (E) Frontal section through a stage 31 wing showing abundant syndecan-3 expression in the perichondrium of the skeletal elements. (F,G) Bright-field and corresponding dark-field autoradiographs of *in situ* hybridizations of frontal sections through the radius of a stage 31 wing demonstrating the presence of syndecan-3 transcripts along the periosteum of the developing long bone. Incubation in primary antisera 46–92 at 1:100 was performed for 1 hr at room temperature. Scale bars in A–D represent 500 mm and 1000 mm in E.

FIG. 9. (A) Frontal section of a Day 12 wing. (B,C) Higher magnification (4×) of A showing abundant syndecan-3 expression along the periosteum of a long bone and syndecan-3 expression in the dermal condensations of an early feather bud, respectively. Bone matrix (b) is indicated in B. Incubation in primary antisera 46–92 at 1:100 was performed for 1 hr at room temperature. Scale bar represents 1000 mm.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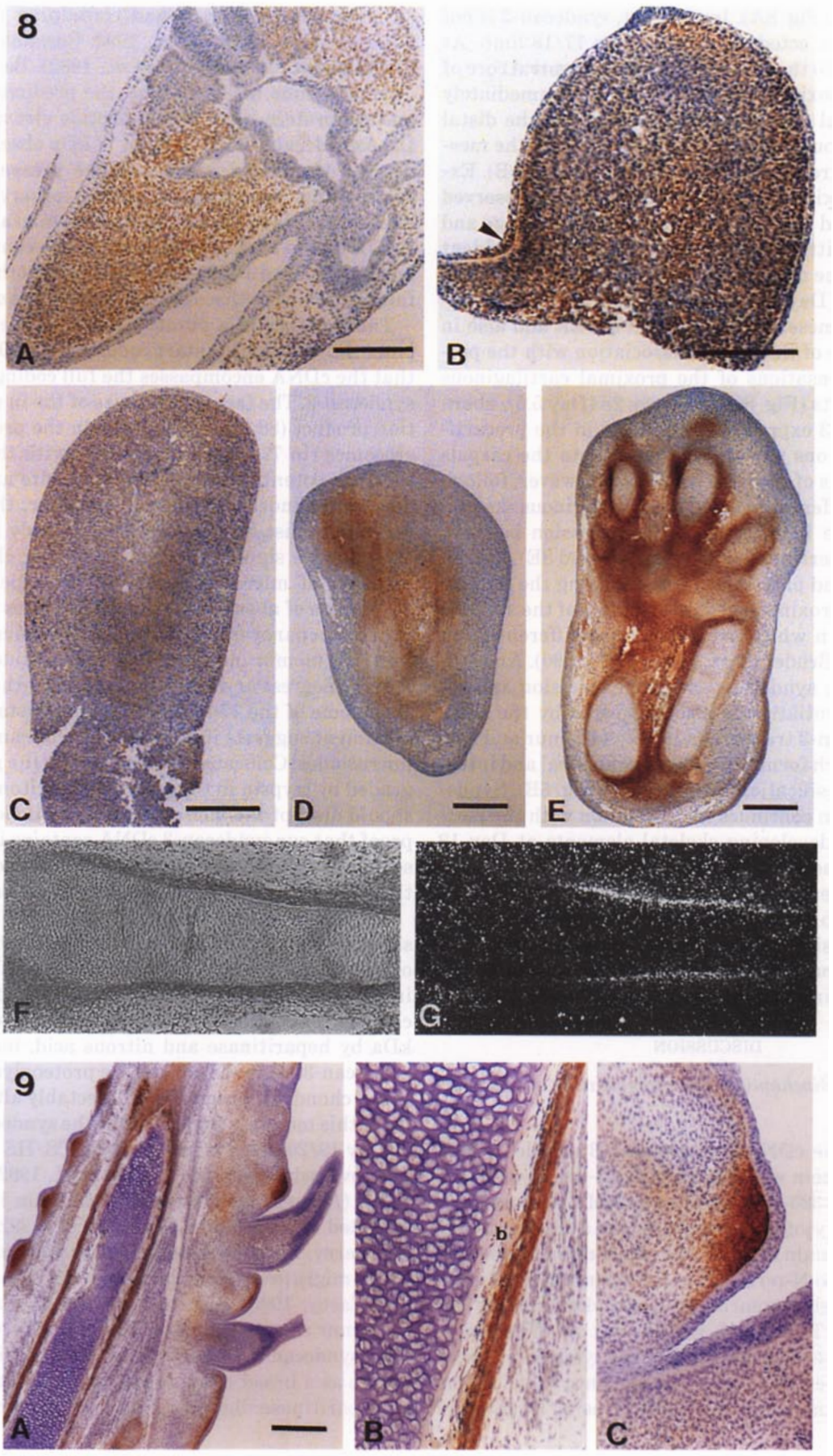
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of the limb bud (Fig. 8A). In contrast, syndecan-3 is not detectable in the ectoderm of the stage 17/18 limb. At stage 22 (Day 3.5) the mesenchyme in the central core of the limb, the proximal ventral region, and immediately under the apical ectodermal ridge (AER) at the distal tip of the limb bud stains more intensely than the mesenchyme in the remainder of the limb bud (Fig. 8B). Expression in proximal ventral ectoderm is also observed at this stage and appears to be specific to this stage and location, since little or no ectodermal staining is evident in the limb at the other stages examined.

At stage 25 (Day 5) syndecan-3 continues to be expressed in the mesenchyme under the AER and also in the central core of the limb in association with the precartilage condensations of the proximal cartilaginous skeletal elements (Fig. 8C). At stage 28 (Day 5.5), abundant syndecan-3 expression is present in the precartilage condensations which will give rise to the carpals and metacarpals of the limb (Fig. 8D). However, following the overt differentiation of the cartilaginous skeletal elements of the limb, syndecan-3 expression becomes limited to the perichondrium (Figs. 8D and 8E). Syndecan-3 mRNA and protein are present along the mid-diaphysis of the proximal skeletal elements of the stage 31 wing, an area in which osteoblasts are differentiating (Figs. 8E–8G) (Bruder and Caplan, 1989, 1990). An association between syndecan-3 mRNA expression and osteogenic differentiation is also suggested by the presence of syndecan-3 transcripts in Day 14 femur and Day 14 calvaria which form through endochondral and intramembranous ossification, respectively (Fig. 5B). Syndecan-3 expression continues in association with the periosteum of the developing skeletal elements at Day 12 (Figs. 5B, 9A and 9B). Abundant expression can also be seen in the mesenchyme of the early feather buds in the same stage limb (Figs. 9A and 9C). This intense mesenchymal expression is largely lost at later stages of feather development, suggesting a transient role for syndecan-3 during feather formation (Fig. 9A).

DISCUSSION

Molecular and Biochemical Characterization of Chicken Syndecan-3

Our composite cDNA for syndecan-3 encodes a 405-amino-acid protein which includes a 22-amino-acid signal sequence, a 325-amino-acid extracellular domain, a 25-amino-acid cytoplasmic domain, and a 33-amino-acid cytoplasmic domain (Fig. 2). The ectodomain can be further divided into N-terminal and C-terminal GAG clusters totaling eight potential GAG attachment sites and an intervening T–S–P-rich region (Fig. 2). When compared with other known syndecans, syndecan-3 most closely resembles syndecan-1 in sequence (37% amino acid identity) and in overall structure, being larger than either syndecan-2 or -4 and containing two separate GAG clusters (David *et al.*, 1993; Bernfield *et al.*, 1992; Kojima *et al.*, 1992; Pierce *et al.*, 1992). Based upon the deduced amino acid sequence, the predicted size of the mature protein after signal peptide cleavage is 40,759 Da, considerably smaller than 77 kDa observed for syndecan-3 translated *in vitro* in the presence of microsomes. This discrepancy between observed and predicted sizes may be due to anomalous migration on SDS–PAGE due to high alanine and proline content. Similar anomalies have been observed for all other syndecan family members (discussed in Bernfield *et al.*, 1992).

The presence of a putative signal sequence near the potential methionine start codon of our cDNA suggests that the cDNA encompasses the full coding sequence of syndecan-3. The fact that the size of the *in vitro* translation product (88 kDa) is reduced in the presence of microsomes (to 77 kDa) is consistent with the possibility that the potential translation start site and signal sequence are indeed functional. However, this latter observation must be interpreted cautiously since, if only the putative signal sequence was being cleaved in the presence of microsomes, one might expect a product with a size of about 85 kDa, not 77 kDa, as we observed. This discrepancy may reflect the anomalous slow migration of membrane-spanning polypeptides on SDS–PAGE (Segrest and Jackson, 1972), and the fact that at least some of the 77-kDa product is resistant to trypsin treatment suggests it has indeed been translocated into microsomes. Consistent with this view the product is degraded by trypsin in the presence of Triton X-100 which should disrupt the microsomes. Nevertheless, definitive proof that our syndecan-3 cDNA contains its full coding sequence will require future studies aimed at mapping the transcriptional start site of the syndecan-3 mRNA.

Polyclonal antibodies produced against a purified fusion protein containing the C-terminal 186 amino acids of syndecan-3 recognize a broad band of about 170 kDa in Western blots of extracts from Day 19/20 embryonic chicken brain. This band is reduced to a size of about 120 kDa by heparitinase and nitrous acid, indicating that syndecan-3 is a heparan sulfate proteoglycan. The failure of chondroitinase ABC to detectably alter the mobility of this molecule suggests that the syndecan-3 present in Day 19/20 brain is not a hybrid CS/HS proteoglycan as previously suggested (Gould *et al.*, 1992). Rat syndecan-3 (*N*-syndecan) isolated from brain has also been reported to be a HSPG (Carey *et al.*, 1992; Chernousov and Carey, 1993). Syndecan-3 isolated from neonatal rat brain migrates at approximately 250 kDa (Chernousov and Carey, 1993) and that from chick brain reported here runs at approximately 170 kDa. We have observed that syndecan-3 isolated from Day 6–7 limb buds migrates as a broad band at approximately 250 kDa prior to heparitinase digestion (data not shown), suggesting

that syndecan-3 may be polymorphic from tissue to tissue with respect to the number and or size of GAG chains present as has been demonstrated for syndecan-1 (Sanderson and Bernfield 1988; Boutin *et al.*, 1991; Brauker *et al.*, 1991).

The increased migration rate of syndecan-3 after treatment with TFMS compared to that treated with heparitinase (110 vs 120 kDa, Fig. 4) suggests that there may be additional carbohydrate present on the core protein that is not susceptible to heparitinase digestion. The presence of a T-S-P or "mucin-like" domain between the N-terminal and C-terminal GAG clusters suggests that syndecan-3 has the potential to receive numerous O-linked oligosaccharide chains which could account for the observed difference in size between the material treated with TFMS and that treated with heparitinase. Furthermore, the bands detectable after TFMS treatment are broad, suggesting incomplete removal of carbohydrate and indicating that 110 kDa cannot be used as a reliable estimate of the size of the mature syndecan-3 core protein, as even small amounts of carbohydrate can have pronounced effects on the migration of glycoproteins on SDS-PAGE (Segrest and Jackson, 1972). Thus, the difference in size between the syndecan-3 core protein produced *in vitro* (77 kDa) and that observed after TFMS treatment of endogenous syndecan-3 (110 kDa) is likely due to incomplete deglycosylation of the core protein by TFMS.

Syndecan-3 Expression during Embryogenesis

Syndecan-3 exhibits spatially discrete and developmentally regulated expression patterns in the chick embryo, suggesting an involvement in the morphogenesis and differentiation of several organ systems. Although our biochemical and expression studies do not allow us to assign definitive functions to syndecan-3, our observations are consistent with the hypothesis that syndecan-3, like other members of the syndecan family, may function as an extracellular matrix receptor and/or as a coreceptor for growth factors such as FGF (Bernfield *et al.*, 1992; Chernousov and Carey, 1993; David *et al.*, 1992).

Syndecan-3 exhibits abundant expression in the brain and neural tube of the chick embryo, suggesting that it may play an important role in the development of the central nervous system. At Day 20, syndecan-3 is expressed essentially uniformly throughout the brain of the chick embryo, similar to its expression pattern in neonatal rat brain (*N*-syndecan, Carey *et al.*, 1992). Chernousov and Carey (1993) have recently demonstrated that syndecan-3 isolated from neonatal rat brain selectively binds FGF-2 (bFGF) and have suggested that syndecan-3 may function as a coreceptor for FGF-2 during the development of the central nervous system. This may also be true in the chicken as we have found that syndecan-3 exhibits a striking expression in the floor plate of the neural tube of the chick embryo, an organizing or signaling center that appears to be important in the organization of the nervous system (Yamada *et al.*, 1993). Nurcombe *et al.* (1993) have recently suggested that a polymorphic heparan sulfate proteoglycan may regulate the activity of FGF in the floor plate of the mouse central nervous system, raising the possibility that syndecan-3 plays a similar role in the floor plate of the chick embryo. It is also noteworthy that syndecan-3 is enriched in the posterior portion of the developing chick lens adjacent to the vitreous and in the mesenchymal component of early feather buds, both regions in which FGFs have been implicated in growth and patterning (Schulz *et al.*, 1993; Noji *et al.*, 1993). The abundant neural distribution of syndecan-3 reported here is also consistent with the recent finding of Raulo *et al.* (1994) that rat syndecan-3 (*N*-syndecan) serves as a receptor for HB-GAM, a molecule abundant in neonatal rat brain that may be involved in neurite extension.

Syndecan-3 is expressed in the developing chick limb in a fashion consistent with its possible involvement in several aspects of limb morphogenesis and differentiation. During early limb development, syndecan-3 is expressed by the distal mesenchymal cells of the limb bud that are undergoing proliferation in response to the AER. Several recent studies indicate that outgrowth and patterning of limb mesenchyme in response to the AER are mediated at least in part by members of the FGF family (Fallon *et al.*, 1994; Niswander and Martin, 1993; Niswander *et al.*, 1993; Riley *et al.*, 1993). Syndecan-3 expressed by the subridge mesenchyme of the limb bud may therefore play an essential role in limb outgrowth, serving as a low affinity receptor for FGF. Our studies also suggest that syndecan-3 may be involved in limb cartilage differentiation. We have found that syndecan-3 is transiently expressed during the formation of precartilage condensations of the skeletal elements of the limb and therefore may serve as a cell-cell or cell-matrix receptor involved in this critical condensation process (see Gould *et al.*, 1992, for a detailed discussion). Significantly, we also observe syndecan-3 expression in the sclerotome which consists of prechondrogenic cells destined to give rise to the framework of the axial skeleton. Following the condensation phase of chondrogenesis, detectable syndecan-3 expression becomes limited to the perichondrium of the cartilaginous skeletal elements of the limb and subsequently is expressed in association with the differentiating osteoblasts of the periosteum. The expression of transcripts for syndecan-3 in calvaria, which form through intramembranous bone involving no cartilage intermediate, as well as its localized expression in the periosteum of developing long bones strongly suggests an association between

syndecan-3 expression and osteoblast differentiation. Thus, syndecan-3 may play important roles in both chondrogenesis and osteogenesis.

Although syndecans 1, 2, and 3 share similar structures, it is noteworthy that the pattern of expression of syndecan-3 in the chick embryo differs considerably from the expression patterns of syndecan-1 (Trautman *et al.*, 1991; Bernfield *et al.*, 1992) and syndecan-2 (fibroglycan, David *et al.*, 1992) in the mouse embryo. Neither syndecan-1 nor syndecan-2 are expressed in the developing central nervous system (CNS) (Bernfield *et al.*, 1992; David *et al.*, 1992), whereas syndecan-3 is expressed in the CNS of both the rat (Carey *et al.*, 1992; Chernousov and Carey, 1993) and the chick embryo. Interestingly, syndecan-1 and syndecan-3 appear to exhibit complementary patterns of expression in some developing systems. For example, syndecan-3 is expressed in the placodal epithelia of the developing otic and lens vesicles but not by preplacodal ectoderm. Conversely, syndecan-1 is expressed by preplacodal ectoderm, and its expression ceases at the time of placode formation (Trautman *et al.*, 1991). In the developing limb, syndecan-3 is expressed within precartilage condensations during the early phases of chondrocyte differentiation whereas syndecan-1 expression in the central region of the mouse limb bud ceases at the onset of chondrogenesis (Solursh *et al.*, 1990). The expression patterns of syndecan-3 in chick embryo and syndecan-2 in mouse embryo (David *et al.*, 1992) appear more similar, exhibiting several common sites of expression including the head mesenchyme, the branchial arch mesenchyme, the sclerotome, and the perichondrium and periosteum of developing skeletal elements. However, expression of syndecan-2 is restricted to mesenchymal tissues with little or no expression detected in ectodermal derivatives (David *et al.*, 1992), unlike the expression patterns noted here for syndecan-3. Thus although syndecan-1, -2, and -3 would appear to have potential for redundancy of function, they often exhibit mutually exclusive and/or reciprocal expression domains during embryogenesis. David *et al.*, 1992 have suggested that, despite their structural similarities, the different syndecans may assume different functions and/or may have similar functions but operate in separate temporal or spatial contexts.

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